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Biosensor based on the biocatalysis of microperoxidase-11 in nanocomposite material of multiwalled carbon nanotubes/room temperature ionic liquid for amperometric determination of hydrogen peroxide

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ABSTRACT

A novel nanocomposite material of multiwalled carbon nanotubes (MWCNTs) and room temperature ionic liquid (RTIL) *N*-butylpyridinium hexafluorophosphate ($BPPF_6$) was explored and used to construct a novel microperoxidase-11 (MP-11) biosensor for the determination of hydrogen peroxide (H_2O_2). Cyclic voltammetry (CV) and differential pulse voltammetry (DPV) were used to characterize the performance of the biosensor. Under the optimized experimental conditions, H_2O_2 could be detected in a linear calibration range of 0.5 to 7.0 $\times 10^{-7}$ mol L^{-1} with a correlation coefficient of 0.9949 ($n = 9$) and a detection limit of 3.8 $\times 10^{-9}$ mol L^{-1} at 3σ. The modified electrodes displayed excellent electrochemical response, high sensitivity, long-term stability, and good bioactivity and selectivity.

Hydrogen peroxide (H_2O_2)[1] is harmful for biological systems and appears to be involved in the neuropathology of central nervous system diseases [1], has an indirect impact on the acid rain procedure that is harmful to the atmosphere [2], and so on. So, the determination of H_2O_2 is of considerable importance in both clinical and environmental fields. Different from conventional analytical methods such as titrimetry and colorimetry, amperometric biosensors are especially promising because of their simplicity, high sensitivity, and good selectivity. To improve the performance and long-term stability of the enzyme electrode, effective immobilization of enzymes onto the transducer surface through suitable matrix is of great significance [3–5].

Nanocomposite materials as the matrix have attracted researchers' interest recently. The preliminary investigation has demonstrated that such matrix has thermal stability and high conductivity and that the proteins adsorbed on the electrode can still retain their activities [6]. The electrode surface modified with nanocomposite materials usually has good electron transfer efficiency between the matrix electrode and redox proteins such as enzymes. This modification is helpful in developing biosensors and the related apparatus.

Carbon nanotubes (CNTs) as a type of nanomaterials have attracted attention during recent years [7–11]. Many studies have demonstrated that CNTs have excellent electrocatalytic abilities and antifouling properties and, thus, can extend the electronic conduction pathways, can increase the effective surface area, and have the ability to promote electron transfer reactions when used as an electrode material in electrochemical reactions [12–17]. CNTs can be coupled to enzymes to act as electrical connectors between the redox center of the enzymes and the electrodes [18]. Recently, researchers have mixed ionic liquids with carbon materials to form nanocomposite materials and obtained high sensitivity, good biocompatibility and so on [19,20].

Room temperature ionic liquids (RTILs) are compounds consisting entirely of ions that exist in the liquid state around room temperature [21,22]. As replacements for classic molecular solvents in fundamental research and applications, RTILs have good chemical and physical properties such as good chemical and thermal stability, negligible vapor pressure, good electrical conductivity, and a wide electrochemical window [23–27].

Aida's group [28] mixed imidazolium ion-based RTIL with pristine single-walled CNTs to form gels by grinding. This nanocomposite material showed substantial enhancement in dynamic hardness and good electroconductivity. So, it could be used to improve the performance of electrodes. Zhu and coworkers [29] used

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[1] *Abbreviations used:* H_2O_2, hydrogen peroxide; CNT, carbon nanotube; RTIL, room temperature ionic liquid; MWCNT, multiwalled CNT; $OMIMPF_6$, 1-octyl-3-methylimidazolium hexafluorophosphate; GCE, glassy carbon electrode; DA, dopamine; $BPPF_6$, *N*-butylpyridinium hexafluorophosphate; MP-11, microperoxidase-11; MW, molecular weight; OAP, *O*-aminophenol; PB, phosphate buffer; CV, cyclic voltammetry; SEM, scanning electron microscopy; DMF, *N,N*-dimethylformamide; GC, glassy carbon; DPV, differential pulse voltammetry; HRP, horseradish peroxidase; PtE, Pt microelectrode; HB, hemoglobin; SA, sodium alginate; CILE, carbon ionic liquid electrode; IL, ionic liquid; MSF, mesocellular siliceous foam.

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a gel containing multiwalled CNTs (MWCNTs) and RTIL of 1-octyl-3-methylimidazolium hexafluorophosphate ($OMIMPF_6$) to modify a glassy carbon electrode (GCE) as a novel electrode for sensitive determination of dopamine (DA) in the presence of ascorbic acid and uric acid. The thickness of the modified layer was difficult to control and had great impact on the electrochemical properties. MWCNTs and RTIL of *N*-butylpyridinium hexafluorophosphate ($BPPF_6$) mixed solution, which was used instead of the MWCNTs/$OMIMPF_6$ gel, could avoid this problem in our experiment.

In this study, our approaches to development of new kinds of enzyme biosensor for determination of H_2O_2 are essentially based on assembling microperoxidase-11 (MP-11) onto a MWCNTs–$BPPF_6$ nanocomposite material-modified GCE by adsorption. The prepared biosensor exhibited an especially low detection limit, high selectivity, and quite long-term stability (60 days). The developed biosensor works especially at low concentrations of H_2O_2, and there was no interference that might lead to false H_2O_2 measurements. The simple method by entrapping enzyme into biocompatible MWCNTs and RTIL composite provided an effective means for realizing direct electrochemistry of enzyme and for fabricating the novel biosensor. It may be widely used in chemical, biological, clinical, food, and environmental fields.

Materials and methods

Reagents

The following materials were obtained commercially and used as received: MP-11 (~90% pure, molecular weight [MW] 1881, Sigma–Aldrich, USA), H_2O_2 (analytical grade, Shanghai Chemical, China), $BPPF_6$ (97% pure, melting point 65 °C, Hangzhou Kemer Chemical, China), and *O*-aminophenol (OAP, Beijing Hengyezhongyuan, China). MWCNTs (diameter ~10–20 nm and length ~50 μm) with carboxylic groups purchased from Shenzhen Nanotech Port (Shenzhen, China) were purified according to Ref. [30]. All solutions were prepared with doubly distilled water. The other chemicals were of analytical grade and used without further purification. The 0.1-M phosphate buffer (PB) solutions at various pH values were prepared by mixing the stock solutions of NaH_2PO_4 and Na_2HPO_4 and then adjusting the pH with 0.1 M NaOH and H_3PO_4. $Fe(CN)_6^{3-/4-}$ solutions (5.0 mM, containing 0.1 M KCl) with equal concentrations of $Fe(CN)_6^{3-}$ and $Fe(CN)_6^{4-}$ were prepared as electrochemical probes.

Apparatus

Cyclic voltammetry (CV) was performed on a CHI 832B electrochemical analyzer (Shanghai Chenhua Instrument, China) with a three-electrode system composed of a platinum wire as auxiliary electrode, an Ag/AgCl/KCl (saturated) electrode as reference electrode, and a bare or modified GCE as working electrode. All of the electrochemical experiments were performed at an ambient temperature of 20 ± 2 °C. All experimental solutions were deoxygenated by bubbling nitrogen for 30 min, and a nitrogen atmosphere was kept over the solutions during measurements. Scanning electron microscopy (SEM) measurements were carried out on a JSM-6700F scanning electron microscope (Japan Electro, Japan).

Preparation of MP-11/Nafion/MWCNTs–$BPPF_6$/GCE

Prior to use, the glassy carbon electrode was carefully polished with polishing paper and 1.0, 0.3 and 0.05 mm alumina slurry sequentially then dried in air after being washed ultrasonically in water ethanol. Then 2.0 mg of MWCNTs and 14.059 mg of $BPPF_6$ were added into 1 ml of *N*,*N*-dimethylformamide (DMF) solution as the stock solution. After approximately 40 min of sonication, uniformly dispersed MWCNTs and $BPPF_6$ were formed. Then 5 μl of the prepared suspension was dipped onto GCEs to obtain the MWCNTs–$BPPF_6$/glassy carbon (GC)-modified electrodes. After being air-dried, the MWCNTs–$BPPF_6$-modified electrodes were immersed into the aqueous solution of MP-11 (0.8 mg ml^{-1}) for 10 h. The electrodes (denoted as MP-11/MWCNTs–$BPPF_6$-modified electrodes) were then rinsed thoroughly with distilled water to remove the nonadsorbed MP-11. Then 5 μl of Nafion (1 wt%) solution was dipped onto modified electrodes and dried in air to obtain the MP-11/Nafion/MWCNTs–$BPPF_6$-modified electrodes. The modified electrodes were stored at 4 °C in PB solution (pH 6.0) in a refrigerator when not in use. Scheme 1 shows the preparation approach.

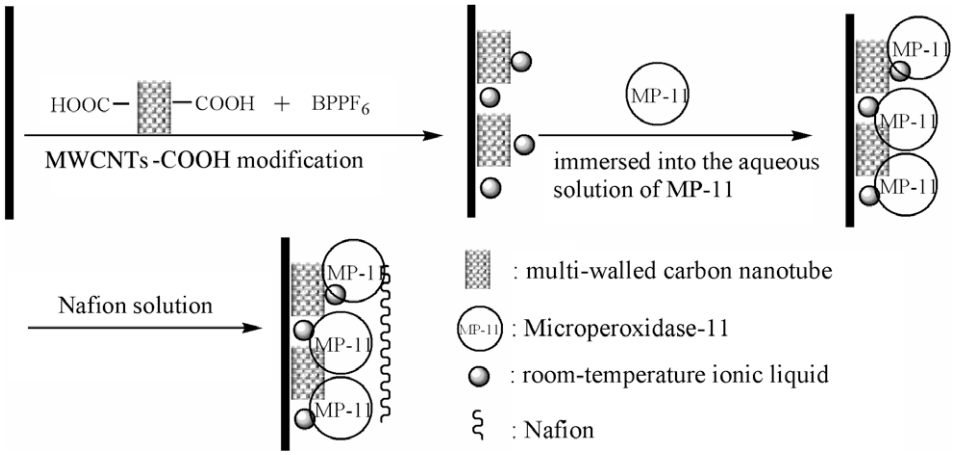
Electrochemical detection

Then 12.5 μl of 0.1 M H_2O_2 was added to 5 ml of 5 mM OAP and 0.1 M PB solution (pH 6.0). The CV curves of the solution were recorded on a CHI 832B electrochemical analyzer with the three-electrode system with a potential scanning range from −0.8 to 0 V.

Results and discussion

Fabrication of H_2O_2 biosensor

The SEM images of GCE, MWCNTs/GCE, and MP-11/Nafion/MWCNTs–$BPPF_6$/GCE are shown Fig. 1. Significant differences in the surface structure of these different electrodes were obtained. Diameters of these bundles ranging from 20 to 50 nm were ob-



Scheme 1. Preparation approach of MP-11/Nafion/MWCNTs–$BPPF_6$/GCE.

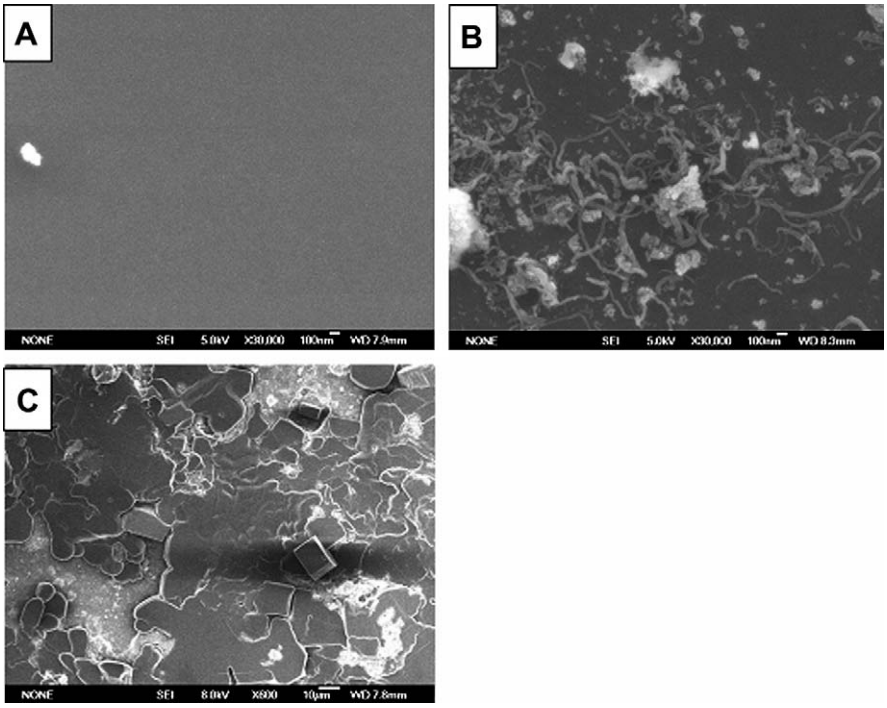


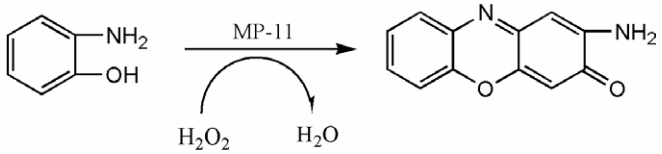
Fig. 1. SEM image of the electrodes: (A) GCE; (B) MWCNTs/GCE; (C) MP-11/Nafion/MWCNTs–$BPPF_6$/GCE.

served (Fig. 1B). In the SEM image of MP-11/Nafion/MWCNTs-$BPPF_6$/GCE (Fig. 1C), MP-11 and MWCNTs were enfolded to Nafion membrane, whereas the RTIL $BPPF_6$ was dispersed into them, as crystalloid state, to approximately 10 μm.

CV behavior of modified electrodes

It is well known that peroxidases can catalyze the oxidation reaction of phenols and aromatic amines by H_2O_2 [31,32]. A possible mechanism of H_2O_2 catalyzed by MP-11/MWCNTs–$BPPF_6$-modified film is postulated as shown in Scheme 2. Fig. 2 shows the CV of various electrodes in 1.0 M KCl solution containing 5.0×10^{-3} M $K_3Fe(CN)_6$ at a scan rate of 20 mV s^{-1}. To discern the role of individual components and possible synergy between them, bare GCE, MWCNTs/GCE, and MWCNTs–BPPF6/Nafion/GCE were studied in 5.0 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ containing 0.1 M KCl at 0.02 V s^{-1}. Bare GCE (Fig. 2A) gave stable and well-defined redox peaks at 119 mV (E_{pc}) and 184 mV (E_{pa}), which was the characteristic of $[Fe(CN)_6]^{3-/4-}$ redox couple in the solution. After coating with MWCNTs and MWCNTs–$BPPF_6$ nanomaterials, the current was increased substantially and still displayed a steady-state diffusion plateau that amplified the peak current of $Fe(CN)_6^{3-/4-}$ redox couple 2.0 and 2.7 times, respectively. So, we chose the coupled MWCNTs and $BPPF_6$ as modified material. The synergistic effects can be ascribed to the excellent electron transfer ability of MWCNTs and $BPPF_6$.

The redox active center, an iron protoporphyrin IX, in MP-11 is not shielded by a polypeptide; hence, electron transfer can easily be achieved under appropriate conditions. Fig. 3 shows the CV of various electrodes in different solutions. No response of the GCE was observed in the absence of OAP and H_2O_2 in 0.1 M PB solution (pH 6.0) (curve a). When 5.0 mM OAP was added to the PB solution, the CV of MP-11/MWCNTs–$BPPF_6$/GCE showed redox peaks at −0.260 and −0.235 V (curve b). When 5.0 mM OAP and 0.25 mM H_2O_2 were added to the PB solution, the CV of MP-11/Nafion/MWCNTs-modified electrodes showed a couple of stable and well-defined oxidation and reduction peaks for OAP at −0.301 V and −0.230 V (curve c), but significant redox peaks appeared at −0.260 and −0.235 V (curve d) when the working electrode was changed to MP-11/Nafion/MWCNTs–$BPPF_6$/GCE. The result indicates high electrocatalytic activity of the immobilized MP-11 of



Scheme 2. Possible mechanism of H_2O_2 catalyzed by MP-11/MWCNTs–$BPPF_6$-modified film.

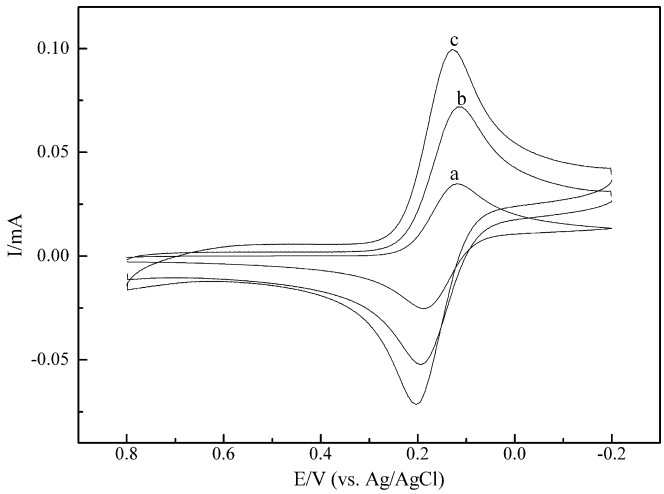


Fig. 2. CV obtained with GCE (a), MWCNTs/GC (b), and MWCNTs–$BPPF_6$/GCE (c) in 1.0 M KCl solution containing 5.0 mM $K_3Fe(CN)_6$ at a scan rate of 20 mV s^{-1}.

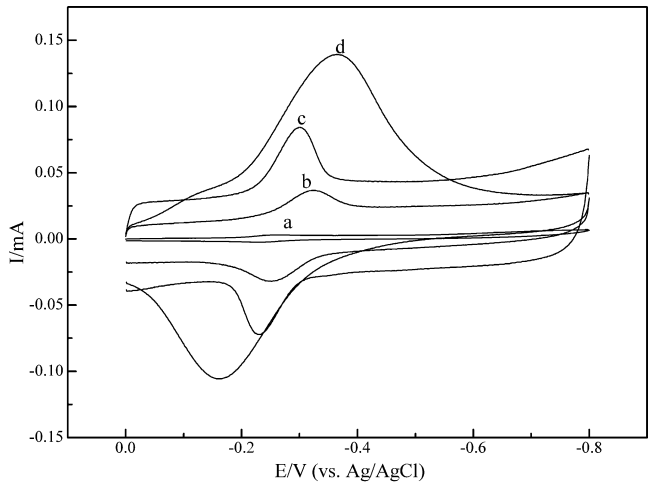


Fig. 3. CV of different electrodes at 0.1 V s^{-1}: (a) GCE in 0.1 M PB solution (pH 6.0); (b) MP-11/Nafion/MWCNTs–$BPPF_6$/GCE in 0.1 M PB solution (pH 6.0) + 5.0 mM OAP; (c) MP-11/Nafion/MWCNTs/GCE in 0.1 M PB solution (pH 6.0) + 5.0 mM OAP + 0.25 mM H_2O_2; (d) MP-11/Nafion/MWCNTs–$BPPF_6$/GCE in 0.1 M PB solution (pH 6.0) + 5.0 mM OAP + 0.25 mM H_2O_2. Scan rate: 0.10 V s^{-1}; quiet time: 2 s.

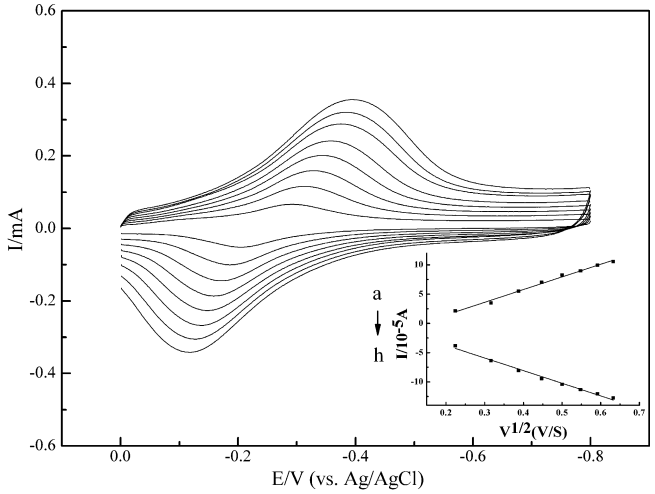


Fig. 4. Typical CV curves of MP-11/Nafion/MWCNTs–$BPPF_6$/GCE in 0.1 M PB solution (pH 6.0) at different scan rates (V s^{-1}): (a) 0.05 V s^{-1}; (b) 0.1 V s^{-1}; (c) 0.15 V s^{-1}; (d) 0.2 V s^{-1}; (e) 0.25 V s^{-1}; (f) 0.3 V s^{-1}; (g) 0.35 V s^{-1}; (h) 0.4 V s^{-1}. The inset shows a calibration plot of peak current versus scan rate.

the biosensor for H_2O_2. The electrochemical reaction corresponds to the conversion of MP-Fe(III) and MP-Fe(II) [33]. The $E^{0'}$ is −0.248 V at 0.1 V s^{-1} of the MP-11/Nafion/MWCNTs–$BPPF_6$/GCE. The value of $E^{0'}$ is similar to that reported for MP-11 assembled on Pt microelectrode modified with CNTs [34]. The number of electrons exchanged (n) could be estimated from the Langmuir film system as follows [35]:

$$Ip = \frac{nFQv}{4RT},$$

where v is the scan rate and Q is the quantity of charge; thus, n could be estimated at 1.05, indicating that the redox reaction of MP-11 on the MWCNTs–$BPPF_6$/GCE belongs to a single electron transfer process.

Influence of scan rate

Typical CV curves of the MP-11/Nafion/MWCNTs–$BPPF_6$/GCE in 0.1 M PB solution (pH 6.0) at different scan rates are shown in Fig. 4. It can be seen that a pair of roughly symmetric anodic and cathodic peaks appeared with nearly equal peak currents in the scan rate range from 0.05 to 0.4 V s^{-1}. The peak-to-peak separation also increased with the scan rate. A good linear relationship was found for the peak current and scan rate, with the results shown in the Fig. 4 inset. The reduction and oxidation peak currents rise linearly with the linear regression equations as I_{pc} (μA) = 21.648v1/2 (V s^{-1})1/2 − 2.8874 (n = 8, γ = 0.9934) and I_{pa} (μA) = −19.696v1/2 (V s^{-1})1/2 − 0.2652 (n = 8, γ = 0.9918), respectively, suggesting that the reaction is a quasireversible diffusion-controlled process.

Optimization conditions for the detection of H_2O_2

The performance of the biosensor depended on the amount of MP-11 immobilized on the MWCNTs–$BPPF_6$/Nafion/GCE because the content of $BPPF_6$ can affects the adsorption of MP-11 on MWCNTs. So, the ratio between MWCNTs and $BPPF_6$ should be chosen. The differential pulse voltammetry (DPV) curves of different amounts of $BPPF_6$ (0.01, 0.02, 0.05, 0.1, 0.5, and 1.0 M) were obtained in 5 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ containing 0.1 M KCl at 0.02 V s^{-1} (Fig. 5A). The results showed that 2 mg of MWCNTs and 14.059 mg of $BPPF_6$ (0.05 M) had the best CV curve. The oxidation and reduction peaks were at 0.101 and 0.184 V, respectively.

The electrochemical detection of H_2O_2 by the above-prepared biosensor also depended on the electrochemical conditions, including the concentration of OAP and the pH value of the substrate solution. When the OAP concentration increased in the PB solution (pH 6.0) containing 0.25 mM H_2O_2, the CV peak current response increased correspondingly and approached a maximum value at a concentration of 5.0 mM (Fig. 5B). Thus, this concentration was chosen as the optimal OAP concentration.

The pH value of the substrate solution effect on the electrochemistry was examined, and the results are shown in Fig. 5C. The biosensor demonstrated the maximum response at a pH value of 6.0 in 0.1 M PB solution containing 5.0 mM OAP and 0.25 mM H_2O_2. Thus, the optimal pH value of the enzymatic reaction was 6.0 and was chosen for all experiments.

Selectivity of developed H_2O_2 biosensor

The selectivity of the developed H_2O_2 biosensor was evaluated by H_2O_2 determinations in the presence of some potentially coexisting compounds of H_2O_2 in biological systems. The current of each interfering substance at a concentration of 10 μM, which was one or two times greater than that in biological systems, was obtained in 0.1 M PB solution containing 5.0 mM OAP and 5.0 μM H_2O_2. Table 1 exhibits the effect of the interferents. The results show that glucose, sucrose, citric acid, uric acid, glycine, and ascorbic acid did not cause any observable interference, suggesting that, especially at low concentrations of H_2O_2, there was no interference by ascorbic acid and the like that might lead to false H_2O_2 measurements.

Electrochemical detection of H_2O_2

The result of MP-11/Nafion/MWCNTs–$BPPF_6$/GCE showed good electrocatalytic activity toward H_2O_2 reduction. The CV signals were obtained under the optimal conditions shown in Fig. 6. With increasing H_2O_2 concentrations, the catalytic reduction peak current of MP-11/Nafion/MWCNTs–$BPPF_6$/GCE increased linearly. The linear response range of the biosensor to H_2O_2 concentration was 0.05 to 0.7 μM, and the linear regression equation was (10^{-5} A) = 10.863 C (10^{-7} M) − 0.4159 (n = 9, γ = 0.9949) with a detection limit of 3.8 × 10^{-9} M at 3σ. The results indicated that MP-11 in Nafion/MWCNTs–$BPPF_6$/GCE had a higher sensitivity to

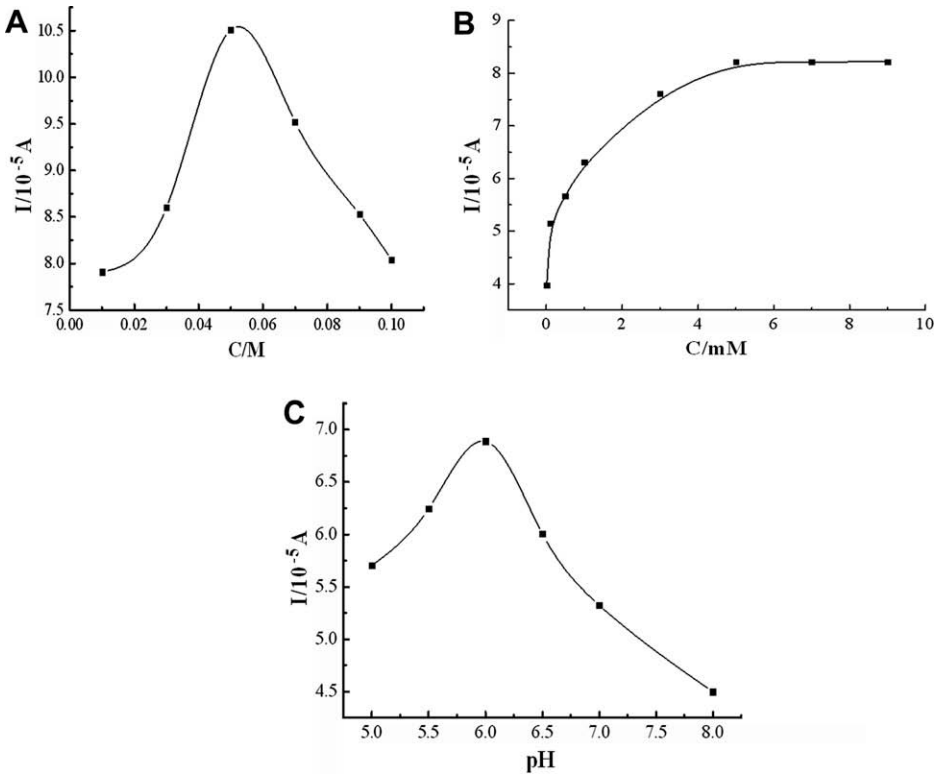


Fig. 5. Effects of pH of buffer solution (A), concentration of $BPPF_6$ (B), and concentration of OAP (C) on the DPV peak current.

Table 1
Selectivity of developed H_2O_2 biosensor.

Potential interfering substance	Current ratio
H_2O_2 (5 μM)	1
Glucose	0.97
Sucrose	0.99
Citric acid	0.98
Uric acid	1.03
Glycine	0.96
Ascorbic acid	0.99

Note. The current of each interfering substance was obtained in 0.1 M PB solution containing 5 mM OAP and 5 μM H_2O_2. The current ratio was the current for mixtures of 10 μM interfering substance and 5 μM H_2O_2 compared with that of 5 μM H_2O_2.

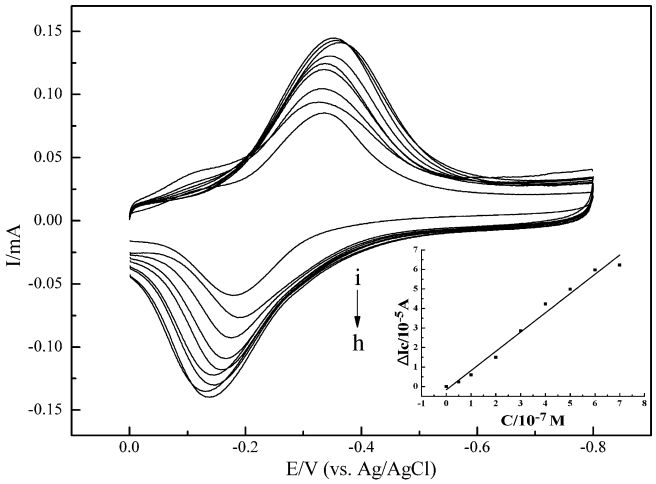


Fig. 6. CV curves obtained with different concentration of H_2O_2 in 5.0 mM OAP and 1.0 M PB solution of MP-11/MWCNTs–$BPPF_6$/Nafion/GCE: (a) 0.5×10^{-7} M; (b) 1.0×10^{-7} M; (c) 2.0×10^{-7} M; (d) 3.0×10^{-7} M; (e) 4.0×10^{-7} M; (f) 5.0×10^{-7} M; (g) 6.0×10^{-7} M; (h) 7.0×10^{-7} M. The inset shows the plot of catalytic oxidation peak current versus the concentration of H_2O_2.

H_2O_2. The modified electrodes display much better electrochemical characteristics, higher sensitivity, and much lower detection limits than those reported previously [36]. In addition, the long-term stability of the MP-11/Nafion/MWCNTs–$BPPF_6$/GCE was investigated over a 60-day period. The catalytic current response could maintain approximately 90% of its original response in 2 months when the modified electrode was stored at 4 °C and measured intermittently.

To demonstrate the performance of the developed H_2O_2 biosensor, a comparison of the linear range, detection limit, and stability obtained by several enzyme-based electrodes for H_2O_2 detection was made in Table 2. The electrodes modified with MWCNTs or RTIL were compared [32,36–39]. The stability of the developed H_2O_2 biosensor was higher than that obtained by horseradish peroxidase (HRP)/MWCNTs/GCE [37] but similar to that obtained by MP-11/MWCNTs/Pt microelectrode (PtE) [32] and hemoglobin (HB)/MWCNTs/grafted collagen matrix [38]. The good storage stability can be attributed to the strong interactions between the MP-11 and MWCNTs, so MP-11 molecules can be firmly immobilized on the surface of the modified electrodes. The detection limit of this developed H_2O_2 biosensor was lower than the detection limits of biosensors based on MP-11/MWCNTs/PtE [32], sodium alginate (SA)–HRP–carbon ionic liquid electrode (CILE) [36], HRP/chitosan/ionic liquid (IL)/GCE [39], and HB/RTIL/mesocellular siliceous foams (MSFs)/GCE [40], and the linear range was wider than that of SA–HRP–CILE [36] but much more narrower than that of HRP/MWCNTs/GCE [37] and HRP/chitosan/IL/GCE [39].The result indicated that the MWCNTs–$BPPF_6$ provided a biocompatible microen-

Table 2
Comparison of developed H_2O_2 biosensor with other biosensors.

H_2O_2 biosensor enzyme electrode	Linear range (μM)	Detection limit (μM)	Stability
MP-11/Nafion/MWCNTs–$BPPF_6$/GCE (this work)	0.05–0.7	0.0038	90%/ 60d
MP-11/MWCNTs/PtE [32]	3.3–38.4	0.7	60d
SA–HRP–CILE [36]	1.0–6.0	0.5	–
HRP/MWCNTs/GCE [37]	4–2000	1	80%/ 14d
HB/MWCNTs/grafted collagen matrix [38]	0.6–30	0.13	95%/ 30d
HRP/chitosan/IL/GCE [39]	0.6–160	0.15	–
HB/RTIL/MSFs/GCE [40]	0.2–28	0.08	–

vironment to keep the bioelectrocatalytic activity after MP-11's adsorption on the surface of the modified electrodes.

Conclusions

To conclude, the new MP-11/Nafion/MWCNTs–$BPPF_6$/GCE for quick determination H_2O_2 was studied. MP-11 was obtained at the MWCNTs–$BPPF_6$ composite film-modified GCE by direct electron transfer and a single electron transfer process between the enzyme and the GCE. H_2O_2 could be detected in a linear calibration range of 5.0×10^{-8} to 7.0×10^{-7} mol L^{-1} with a detection limit of 3.8×10^{-9} mol L^{-1} at 3σ. This new electrode presented a very large current response from electroactive substrates due to its enhanced conductivity and biocompatible interface. The modified electrodes display excellent electrochemical response, high sensitivity, long-term stability, and much lower detection limits.

Acknowledgments

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